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First report and genotyping of *Dientamoeba fragilis* in pet budgerigars (*Melopsittacus undulatus*), with zoonotic importance

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Abstract

The protozoan *Dientamoeba fragilis* is one of the most common parasites in the digestive system of humans worldwide. The host range and transmission routes of *D. fragilis*, including the role of animals, are still ambiguous with few reports from non-human primates, sheep, rodents, pigs, a cat and a dog. In this study, we used microscopic and TaqMan qPCR analyses to investigate *D. fragilis*in 150 faecal samples from pet budgerigars (*Melopsittacus undulatus*) in the Central Anatolia Region of Turkey. *Dientamoeba fragilis* DNA was detected in 32 samples, resulting in a mean prevalence of 21.3%. In microscopic examination, trophozoites/cysts of *D. fragilis* were detected in 13 of 32 qPCR-positive samples. SSU rRNA sequence analyses of the qPCR-positive isolates identified genotype 1 of *D. fragilis* as predominant in budgerigars. Phylogenetic analyses of the SSU rRNA gene region clustered *D. fragilis* genotypes, as well as other trichomonads, in separate monophyletic clusters with bootstrap values ≥79.0. Our study provides the first evidence for the natural host status of pet budgerigars for *D. fragilis*and contributes to the knowledge of the epidemiology of this parasite. The high prevalence of genotype 1 of *D. fragilis* suggests that pet budgerigars are suitable reservoirs for zoonotic transmission. Our findings contribute to an increased awareness and knowledge of *D. fragilis* infections in the context of a one-health approach.

KEYWORDS
budgerigars, *Dientamoeba fragilis*, genotyping, prevalence, Turkey, zoonotic importance

1 | INTRODUCTION

*Dientamoeba fragilis*was first described as an enteric amoeba with unclear pathogenicity in 1918 (Jepps & Dobell, 1918; Beaver et al., 1984). Following recent morphological and molecular studies, the parasite was determined to be phylogenetically close to the flagellated trichomonads (Stark et al., 2016). Despite its unclear pathogenic status, *D. fragilis* has been detected in the stools of patients with asymptomatic and different acute and chronic symptoms, such as abdominal pain, nausea, vomiting, diarrhoea and flatulence (Garcia, 2016). Moreover, clinical signs of the disease have general characteristics, making it difficult to distinguish infections by this parasite from those of many other intestinal pathogens (van Gestel et al., 2019).

The life cycle of *D. fragilis* has not yet been clarified. Trophozoites, the vegetative form that thrives in the gut, were long known as the only described life stage, with transmission likely via the faecal–oral route (Clark et al., 2014). Munasinghe et al. (2013) described

a faecal–oral transmission route in a mouse model by defining a typical cyst form previously unknown. However, the cystic form of *D. fragilis* is rarely identified in human stool samples (Cacciò, 2018). Other researchers (Johnson et al., 2004; Ögren et al., 2013; Röser et al., 2013) suggested that eggs of *Enterobius vermicularis* and *Ascaris lumbricoides* might play a role in transmission as carriers, similar to the relation between *Histomonas meleagridis* and *Heterakis gallinae* (Hess et al., 2015). However, the detection of *D. fragilis* DNA in nematode eggs does not indicate the presence of living organisms. Thus, further data are needed to support or refute transmission via helminth eggs (Stark et al., 2016).

Infections of *D. fragilis* in humans have been reported worldwide including in Turkey (Sivcan et al., 2018; Clemente et al., 2021; Sarzhanov et al., 2021; Yildiz et al., 2021). Although most studies have been conducted in developed countries where health and sanitation are generally good, much less is known for other parts of the world (Barratt et al., 2011). Little information is available about the natural host range of *D. fragilis* except for the human host. *Dientamoeba fragilis* has been reported from a few animal species including non-human primates (Hegner & Chu, 1930; Knowles & Das Gupta, 1936; Myers & Kuntz, 1968; Stark et al., 2008; Lankester et al., 2010; Helenbrook et al., 2015), sheep (Noble & Noble, 1952), rodents (Ogunniyi et al., 2014), pigs (Cacciò et al., 2012) and a cat and a dog (Chan et al., 2016). Two genotypes of *D. fragilis* have been described using isolates mainly from humans, including the common genotype 1 and the rare genotype 2, according to nucleotide differences in the small subunit ribosomal RNA (SSU rRNA) (Cacciò, 2018). However, genotype diversity in animal hosts is not clearly known, with only one report indicating the presence of genotype 1 in pigs (Cacciò et al., 2012).

Further investigations are needed to explore the natural non-human host range of *D. fragilis* and corresponding genotypes related to possible zoonotic transmission. We used microscopic and molecular techniques to investigate the occurrence and distribution of *D. fragilis* in household budgerigars, which are among the most bred pet animals and have close relations with humans. Sequence analyses of the SSU rRNA of detected isolates were also used to reveal genotype profiles that might provide further evidence for zoonotic transmission of *D. fragilis*.

2 | MATERIALS AND METHODS

2.1 | Collection of faecal samples

A total of 150 household budgerigars in several regions of the Central Anatolian Region of Turkey were sampled from March to June 2020. Ethics approval was not required. Because fresh faecal droppings were collected from the cages of birds (not from the animals directly) and divided into two portions. The first portion was transferred directly into sterile stool containers and stored at −20°C until DNA extraction. The second portion was placed in sodium acetate–acetic acid–formalin (SAF) fixative (1:3 ratio) and kept at +4°C until microscopic analysis.

2.2 | DNA extraction, TaqMan qPCR analyses and PCR amplification

Genomic DNA (gDNA) was isolated from faecal samples, using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The final nucleic acids were precipitated in 50 μL elution buffer and stored at −20°C.

The extracted gDNA was subjected to a specific qPCR assay with the primers and a TaqMan probe targeting the 5.8S ribosomal RNA gene region of *D. fragilis*, following the described protocol of Verweij et al. (2007). qPCR analyses were carried out using SsoAdvanced™ Universal Probes Supermix (BioRad, CA, USA) on the CFX Connect™ qPCR Detection System (BioRad, CA, USA).

Positive samples for *D. fragilis* in the qPCR analysis were further analysed by nested PCR for sequence analyses and genotyping. The SSU rRNA gene region of *D. fragilis* was amplified using nested PCR assay with the primer pairs DF1 (5'-CTCATAATCTACTTGGA ACCAATT-3′) and DF4 (5'-CCCCGATTATTCTCTTTGATATT-3′) (Vandenberg et al., 2006), and DF322For (5′-GAGAAGGCGCCTGAGAGATA-3′) and DF687Rev (5′-TTCAT ACTGCGCTAAATCATT-3) (Cacciò et al., 2012). The first PCR reactions were carried out with a final volume of 25 μL, including 12.5 μL of commercial ready-to-use master mix (Dream Taq Hot Start Green PCR Master Mix, Thermo Scientific, USA), 1 μM of each primer and 30 ng of gDNA. For the second PCR, 1 μL of the first PCR product was used as a template. The PCR amplifications were performed in a C1000 Touch Thermal Cycler (BioRad, CA, USA). Cycling conditions for the first PCR were as follows: 95°C for 2 min, followed by 40 cycles, each consisting of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min. The nested PCR was the same, except the annealing temperature was 54°C. The secondary PCR products were separated by 1.5% agarose gel electrophoresis and visualized using Fusion FX Gel Documentation System (Vilber Lourmat, France).

2.3 | Microscopic examination of stool samples

Faecal smears from the counterpart of *D. fragilis* DNA-positive samples in SAF fixative were prepared to investigate the presence of trophozoites and/or cysts of the parasite. The preparations were stained with an iron-haematoxylin dye, as described by Stark et al. (2010). Microscopic examination was performed under an Olympus BX51 light microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP70 (Olympus, Tokyo, Japan) digital camera and imaging software cellSens Standard v.1.13 (Olympus, Tokyo, Japan). Smears were examined for the presence of *D. fragilis* trophozoites or cysts at medium (600×) and high (1000×) magnifications, with approximately 500 fields of view for each slide.

2.4 | DNAsequencing, genotyping and phylogenetic analysis

The secondary SSU rRNA PCR products (366 bp) of *D. fragilis* isolates were sequenced in both directions (Macrogen, Amsterdam, The Netherlands), using the DF322For/DF687Rev primers. Nucleotide sequences were paired, assembled and then subjected to a Blast search using Geneious Prime 2022.0.2 (www.geneious.com) software. For genotyping, the consensus sequences of each isolate were aligned with the reference and other related sequences of *D. fragilis* isolates based on a Blast search using the MAFFT algorithm in Geneious Prime. A phylogeny was inferred using a maximum likelihood (ML) method with genetic distance model GTR + G + I by 1000 bootstrap replicates using PHYML (Guindon et al., 2010).

3 | RESULTS

In total, 32 of 150 faecal samples were positive by qPCR assay for *D. fragilis* DNA, resulting in a mean infection prevalence of 21.3%. Threshold cycles (Ct) of the positive isolates were in the range from 22 to 33. In microscopic analysis, trophozoites/cysts of *D. fragilis* were found in 13 of 32 molecularly positive samples (Figure 1).

The SSU rRNA region of the all-qPCR-positive isolates was successfully amplified with nested PCR assay. The sequence of the SSU rRNA gene region of the isolates indicated genotype 1 of *D. fragilis* (Table 1). Eleven isolates had identical sequences and represented a single isolate (ERU-Dfrag1, GenBank accession: MW130447). Blast analyses of ERU-Dfrag1 indicated whole identity with the isolates reported from humans in Italy, the United Kingdom, Germany and Australia. The SSU rRNA sequences of the remaining 21 isolates were wholly identical to each other and showed one nucleotide substitution (T/C on the 72nd base), compared with ERU-Dfrag1 and reference Genotype 1 of *D. fragilis* (Table 1). This variant was also represented with a single isolate (ERU-Dfrag2, GenBank accession: MW130448). The intraspecific nucleotide difference was 0.3% between ERU-Dfrag1 and ERU-Dfrag2.

Phylogenetic relationships between the identified *D. fragilis* isolates and diverse trichomonad isolates from various regions and hosts are presented in the ML tree (Figure 2). ERU-Dfrag1 and ERU-Dfrag2 isolates formed a monophyletic cluster with the isolates of genotype 1 from humans in several regions. The *D. fragilis* group was closer to the *Parahistomonas* group, with 15.2% genetic difference. This was followed by *Tetratrichomonas*, *Histomonas* and *Tritrichomonas* genogroups, with overall genetic differences of 21.5%, 23% and 23.1%, respectively.

4 | DISCUSSION

Pet birds have socio-economic importance with their genetic and exotic value. However, these animals are potential carriers and/or transmitters of zoonotic diseases, such as chlamydophilosis, salmonellosis and microsporidiosis, and infections with Shiga toxin-producing *Escherichia coli* (STEC) or even highly pathogenic avian influenza A H5N1, which have important consequences for human health (Boseret et al., 2013; Gioia-Di Chiacchio et al., 2016; Deng et al., 2019; Pekmezci et al., 2020). Budgerigars are among the most preferred psittacine birds for housing due to their behaviour and close relationship with humans. We report for the first time that these birds are capable hosts for *Dientamoeba fragilis* and might play a role in the transmission dynamics of dientamoebiasis.

Notwithstanding controversy over transmission modes, *D. fragilis* is probably transmitted primarily via the faecal–oral route (Munasinghe et al., 2013; Clark et al., 2014; Stark et al., 2014). In this context, several investigations (Stark et al., 2008; Cacciò

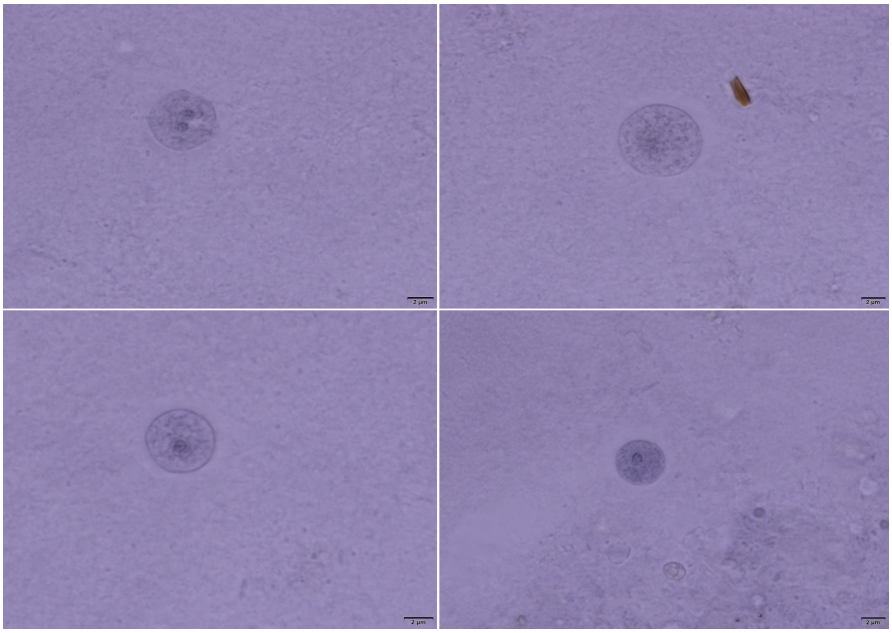

FIGURE 1 *Dientamoeba fragilis*trophozoites/cysts stained with iron-haematoxylin dye (×1000)

TABLE 1 Nucleotide variations (in bold italic characters) in the SSU rRNA gene between our isolates and *D. fragilis* isolates from humans available in GenBank

GenBank accession no	Host	Country	Nucleotide substitutions/insertions									
			72	98	109	114	277	307	327	337	338	339
AY730405 Ref. Genotype 1	Human	Australia	T	T	T	A	T	***G***	A	C	–	T
JQ677152	Human	Italy	T	T	***C***	A	T	–	A	C	–	T
MN914083	Human	Germany	T	T	T	A	T	–	A	C	–	T
JQ677148	Human	United Kingdom	T	T	T	A	T	–	A	C	–	T
JQ677147	Human	Italy	T	T	T	A	T	–	A	C	–	T
JQ677149	Human	Italy	T	T	T	A	T	–	A	C	–	T
JQ677150	Human	Italy	T	T	T	A	T	–	A	C	–	T
MW130447 ERU-Dfrag1	Budgerigar	Turkey	T	T	T	A	T	–	A	C	–	T
MW130448 ERU-Dfrag2	Budgerigar	Turkey	***C***	T	T	A	T	–	A	C	–	T
DFU37461 Ref. Genotype 2	Human	USA	T	***A***	T	***T***	***A***	–	***G***	***T***	***A***	***A***

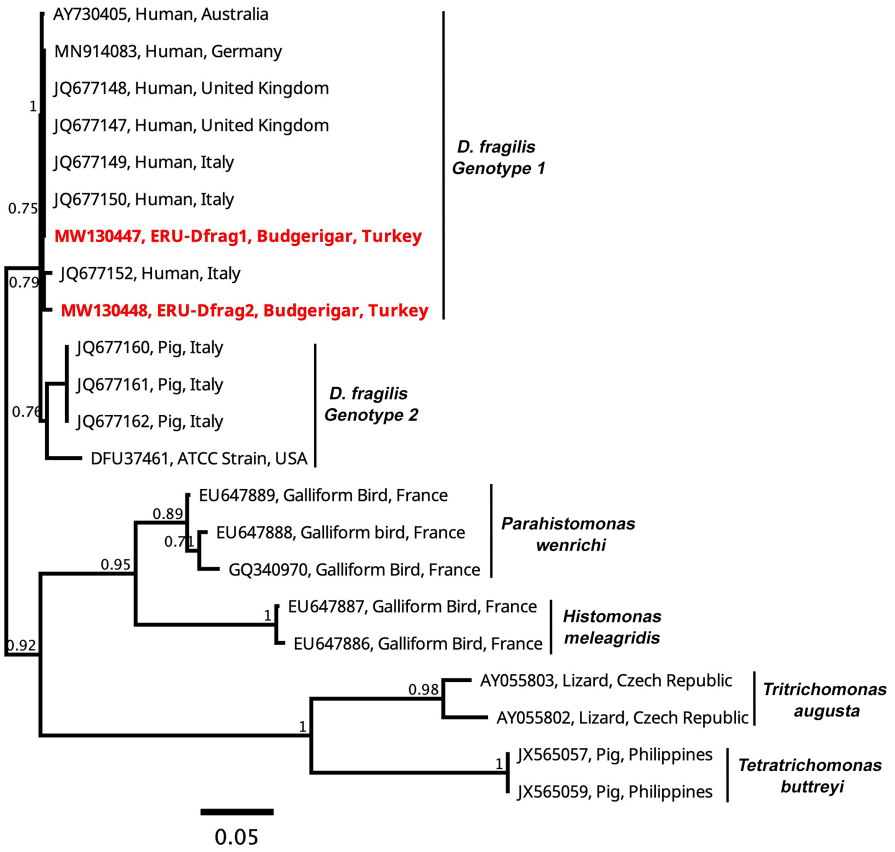

FIGURE 2 Phylogenetic relationships of *D. fragilis* and diverse trichomonad isolates. Maximum likelihood (ML) analyses based on a GTR + G + I model were used in the phylogenetic analyses of the SSU rRNA data set. Isolates included in the data set are given with GenBank accession number, host and country. Isolates characterized in the present study are shown in bold red. The scale bar represents 0.05 substitutions per nucleotide position

et al., 2012; Ogunniyi et al., 2014; Chan et al., 2016) focused on the host status and possible role of different kinds of animals in the zoonotic transmission routes of *D. fragilis*. A previous study (Stark et al., 2008) of faecal samples of wild birds (79 individuals) did not find *D. fragilis*. We identified *D. fragilis* in pet budgerigars, with a relatively high prevalence of 21.3%, using molecular analyses. We also demonstrated the presence of trophozoite/cystic forms in 13 of 32 qPCR-positive samples. These findings suggest that budgerigars are a competent host for *D. fragilis*. We suggest that this high prevalence might be related to the caging of these birds in pet shops, which often involves overcrowding before they are owned by humans. Several studies have demonstrated that overcrowding induces intense stress on pet birds and increases sensitivity to infections (Boseret et al., 2006; Wang et al., 2006; Amonsin et al., 2008), which is possible for the transmission of *D. fragilis* among budgerigars.

The small subunit ribosomal RNA (SSU rRNA) is the first gene used for the characterization of *D. fragilis*, and it has also been used to reveal phylogenetic relationships of trichomonad protozoans (Silberman et al., 1996). In studies using analyses of restriction fragment length polymorphisms of the SSU rRNA gene, two genotypes of *D. fragilis* were characterized and named 1 and 2 (Johnson & Clark, 2000; Peek et al., 2004). The sequence analyses of a 366-bp fragment of the SSU rRNA gene distinguished genotypes 1 and 2 by 8 substitutions, insertions or deletions (Cacciò et al., 2012). Many studies in humans (Peek et al., 2004; Stark et al., 2005; Vandenberg et al., 2006) have targeted this gene region for determining the genotypes of *D. fragilis* and provided evidence for the predominance of genotype 1. Sequence analyses of the corresponding SSU rRNA gene of *D. fragilis* isolates from budgerigars also indicated the presence of the common genotype 1. Single nucleotide variations in genotype 1 sequences were reported previously in a human isolate from Italy (Cacciò et al., 2012). We also identified a single nucleotide polymorphism at the 72nd base (T/C) in a total of 21 isolates, representing ERU-Dfrag2. Sequences of the remaining 11 isolates of *D. fragilis* in budgerigars, representing ERU-Dfrag1, were wholly identical to the common genotype 1 sequences from humans in several countries (Stark et al., 2005; Wylezich et al., 2020; Cacciò et al., 2012). A phylogenetic tree inferred from the ML sequence analyses of the target SSU rRNA gene region clustered the *D. fragilis* genotypes and other trichomonads in separate monophyletic groups. Phylogenetic analyses also revealed the same or close genetic structure of *D. fragilis* in pet budgerigars as the common genotype 1 in humans, suggesting the potential for zoonotic transmission.

5 | CONCLUSION

Our results contribute to the molecular epidemiology and transmission dynamics of *D. fragilis*. The host suitability of budgerigars for this parasite has been revealed for the first time. Considering the widespread hobby breeding of budgerigars and their close contacts with humans, we conclude that this bird species might pose a risk for public health in the context of possible zoonotic transmission dynamics of *D. fragilis*. More detailed studies in various animal species, including pet birds, with large-scale sampling are needed for a better understanding of the host range and zoonotic transmission of *D. fragilis*.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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